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Supplemental Information

A Bmp/Admp Regulatory Circuit Controls Maintenance and Regeneration of Dorsal-Ventral Polarity in Planarians

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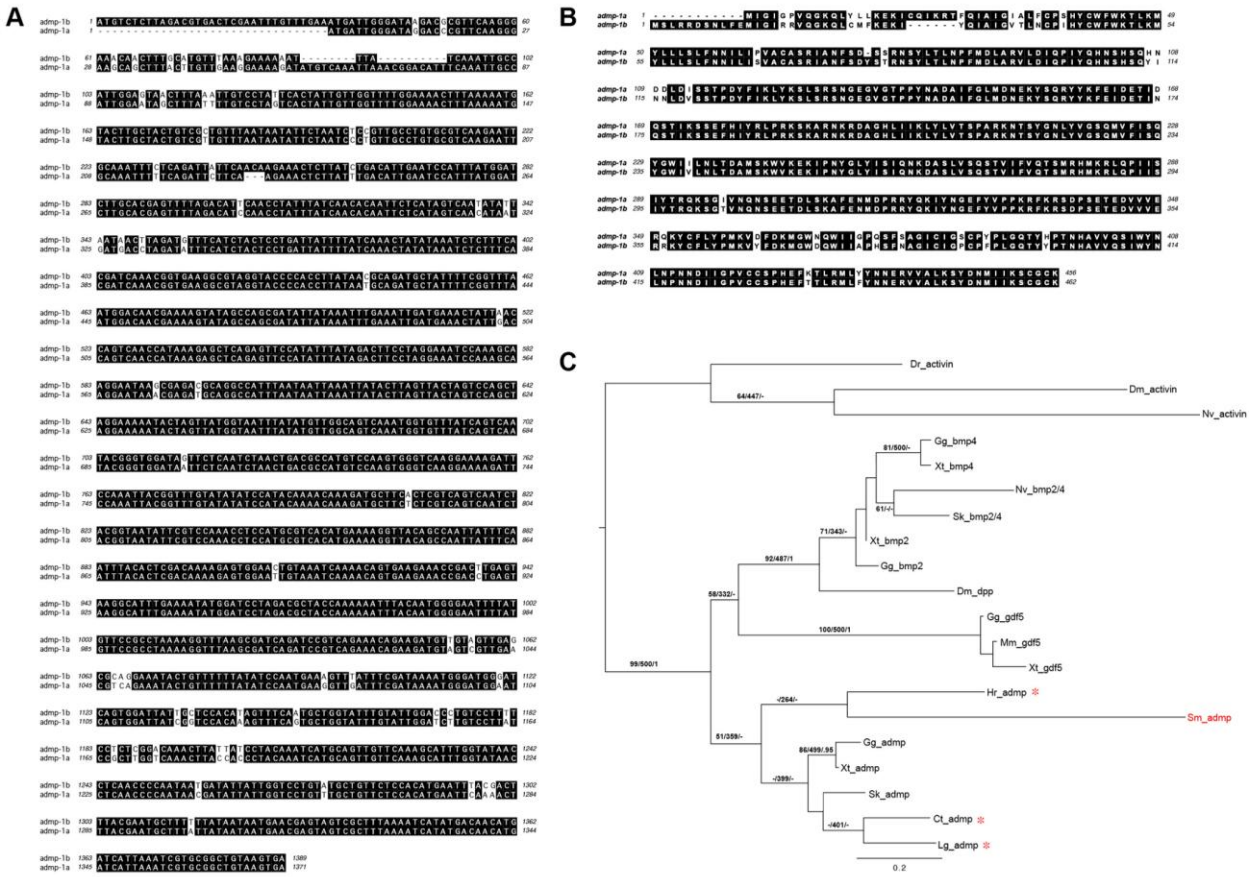

Figure S1. Sequence and Phylogenetic Analysis of *Smed-admp*
(A) Nucleotide alignment of two expressed *S. mediterranea admp* sequences. Due to the high similarity of these sequences, *in situ* hybridization and RNAi directed against either sequence should target both sequences.
(B) Amino acid alignment of proteins encoded by two isolated *admp* cDNA sequences. Sequences were aligned using ClustalW. Identical nucleotides and amino acids are boxed in black. Numbers indicate the nucleotide and amino acid position in (A) and (B) respectively.
(C) Phylogeny of selected TGF beta genes. The maximum likelihood tree based on a ClustalW alignment trimmed with Gblocks is shown here with support values from Likelihood/Neighbor-Joining/Bayesian analyses for the major nodes. *Smed-admp* (red) appears to be fast evolving. Predicted protostome *admp* orthologs are denoted with red asterisks. The phylogenetic position of *Smed-admp*, together with expression and functional data, support orthology with *admp* genes from other organisms. Neighbor-joining values above 250 and Likelihood bootstrap values above 50 are shown, as are Bayesian posterior probabilities above 0.95. Xt = *Xenopus tropicalis*; Gg = *Gallus gallus*; Mm = *Mus musculus*; Sk = *Saccoglossus kowalevskii*; Dm = *Drosophila melanogaster*; Nv = *Nematostella vectensis*; Sm = *Schimdtea mediterranea*; Ct = *Capitella teleta*; Lg = *Lottia gigantia*; Hr = *Helobdella robusta*; Dr = *Danio rerio.*

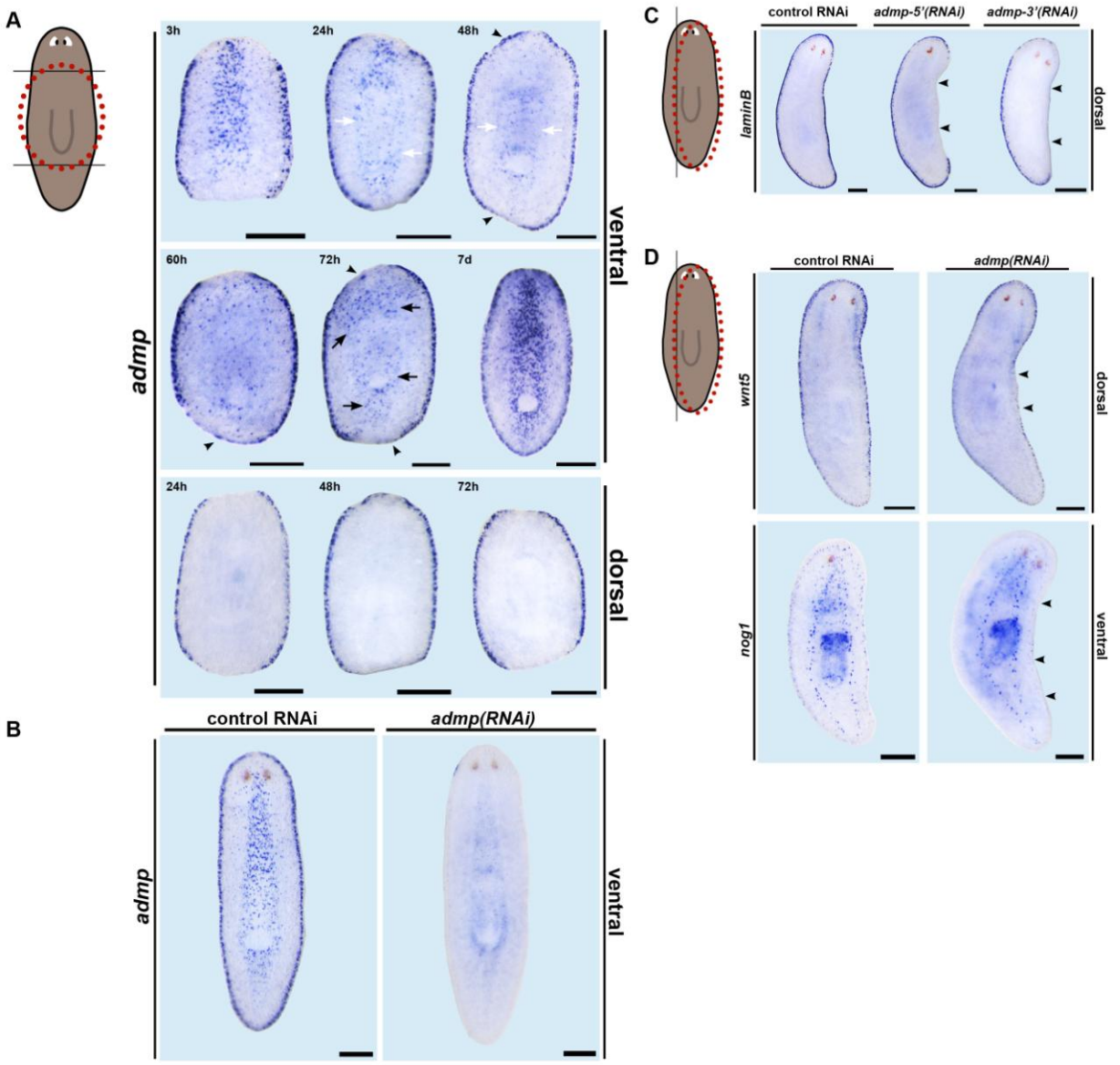

Figure S2. Additional Analyses of *admp* Expression and Knockdown
(A) *admp* expression following head and tail amputation. Expression of *admp* in the ventral domain decreased between 24h and 60h (white arrows) before increasing again at 72h (black arrows). Expression of *admp* in the lateral domain began to return to wound sites at 48h, 60h, and 72h (black arrowheads). At no point during regeneration was dorsal *admp* expression observed.
(B) *admp(RNAi)* animals displayed greatly reduced *admp* expression (n = 6/6).
(C) Animals inhibited for *admp* expression using dsRNAs complementary to either the 5' half or the 3' half of the *admp* gene (*admp-5'* or *admp-3'*, respectively) recapitulated the *admp(RNAi)* lateral regeneration phenotype observed in animals treated with full length dsRNA (n = 6/9 for *admp-5'* and n = 4/8 for *admp-3'*).
(D) *admp(RNAi)* thick fragments failed to regenerate the lateral marker *wnt5* (n = 7/9, black arrowheads) or normal lateral expression of *nog1* (n = 9/9, black arrowheads) 10 days following parasagital amputation. Bars: 200 microns. Anterior, up in all pictures.

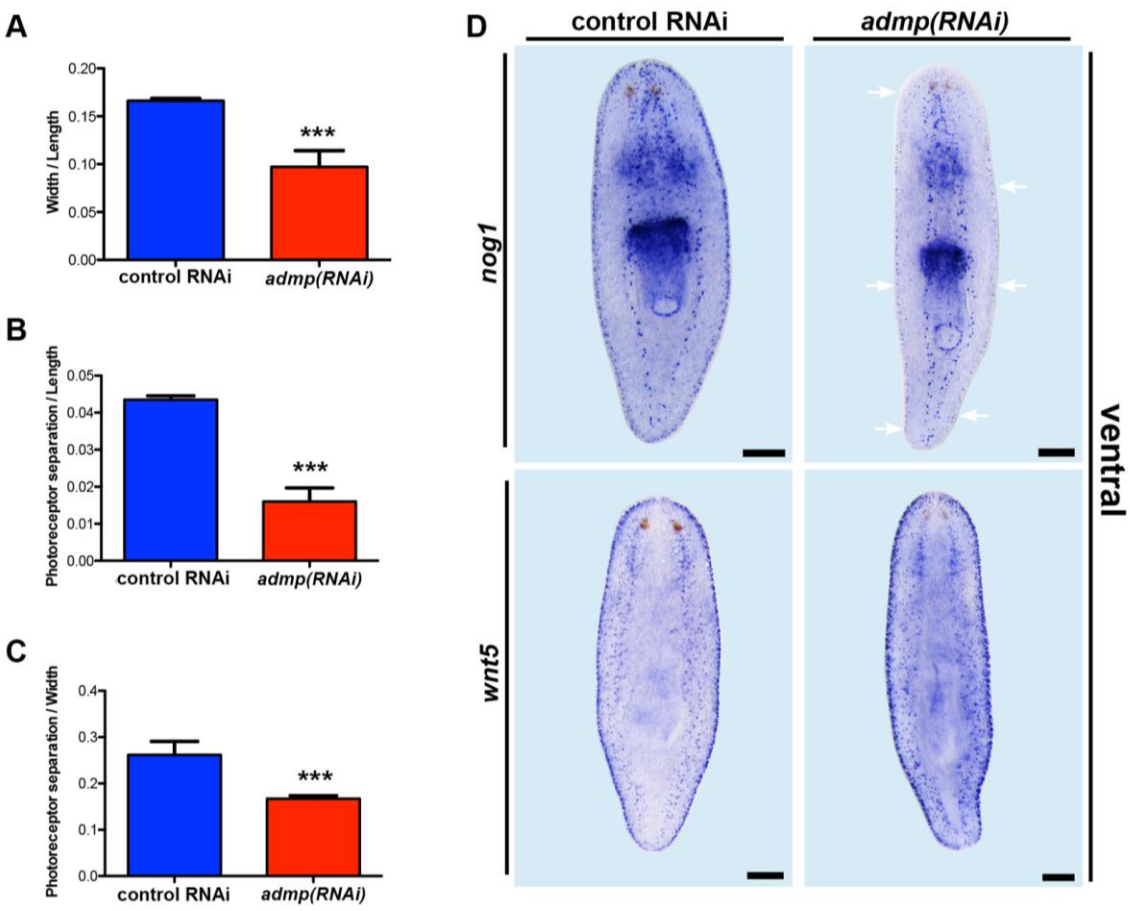

Figure S3. *admp* Is Required for Proper Body Proportion and ML Pattern in Intact, Non-Amputated Animals

(A) Intact *admp(RNAi)* animals were thinner than control animals. Difference was significant, $p < 0.0001$, unpaired t-test.

(B) Intact *admp(RNAi)* animals had more closely positioned photoreceptors than control animals. Difference was significant, $p < 0.0001$, unpaired t-test.

(C) Photoreceptor separation was more greatly affected than total body width as measured at the pharynx in intact *admp(RNAi)* animals. Difference was significant, $p < 0.0001$, unpaired t-test.

(D) Intact *admp(RNAi)* animals displayed no decrease in lateral *wnt5* expression, yet had reduced lateral *nog1* expression (n = 10/10, white arrows) suggesting that the observed effect of *admp* RNAi on *nog1* expression was specific. Measurements were calculated as a ratio with total animal body length in (A) and (B) and of total animal body width in (C). Error bars represent standard deviation in (A-C). Anterior, up in all pictures. Bars: 200 microns.

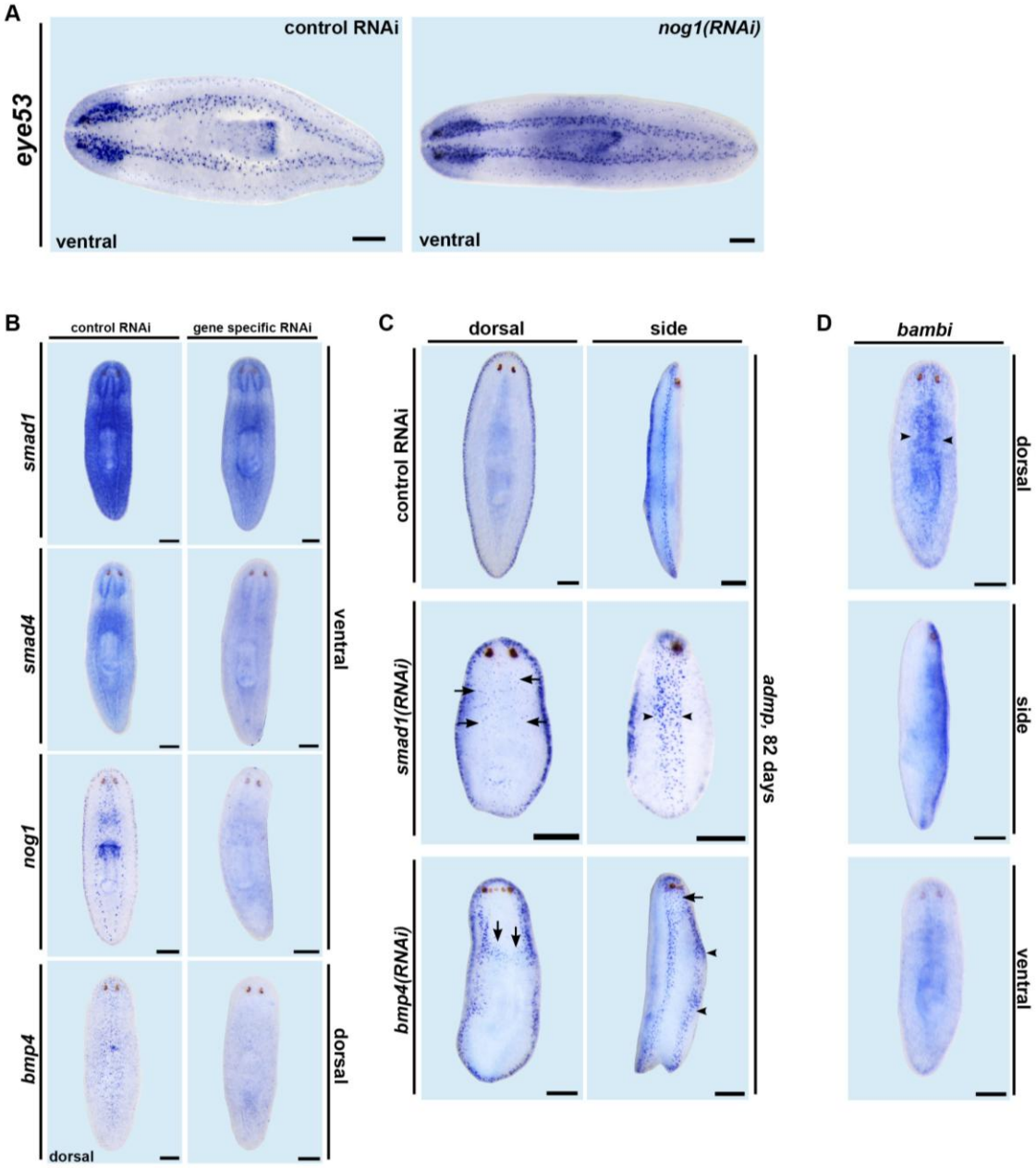

Figure S4. Additional Analyses of Planarian Bmp Pathway Components
(A) Expression of the ventral marker *eye53* is unaffected after 21 days of *Smed-nog1* RNAi in intact non-amputated animals.
(B) Inhibition of Bmp pathway components by RNAi is effective (n > 5 for all).
(C) Inhibition of *smad1* or *bmp4* for 82 days results in dorsally expanded *admp* expression (n = 4/5 and 5/5, respectively, black arrows). Additionally, the lateral domain of *admp* expression is expanded in *smad1(RNAi)* animals (black arrowheads) and duplicated in *bmp4(RNAi)* animals (black arrowheads).
(D) *Smed-bambi* was identified as a putative ortholog of vertebrate *bambi* and is expressed in a broad dorsal domain (black arrowheads). Anterior, left in (A), up in (B-D). Bars: 200 microns.

Supplemental Experimental Procedures

Isolation of *Smed-admp*

A BLAST search was performed on an assembly of the *S. mediterranea* genome (http://genome.wustl.edu) to identify putative *admp* orthologs. Two highly similar *admp* sequences were amplified by PCR from asexual *S. mediterranea* cDNA: *admp-1a* (5'- GATTGGGATAGGACCCGTTC -3' and 5'- TCCCAAGCTAAATACGATTAAAAG -3') and *admp-1b* (5'- TTGGCATTTGGCAATAAATTC -3' and 5'- TCCCAAGCTAAATACGATTAAAAG -3'). Complete gene sequence was determined using 5′ and 3′ RACE PCR (Ambion). An additional highly similar but variant *admp* sequence was identified in sexual *S. mediterranea* genomic sequence. All *admp* experiments were carried out using the *admp-1b* sequence.

RNAi Experiments

PCR was used to amplify the *bmp4* (5'- TTGATGCCAAAGATTCGTTC -3' and 5'- TCAAAATCCCAAGCTAAATACG -3'), *smad1* (5'- TCGTGTTAATTTACCATATTGTTGC -3' and 5'- TGAAGTTAGATTCCACAAGAATAAAGC -3'), *smad4* (5'- GAATTCCTCCAATGGACCAG -3' and 5'- TCCCAAGCTAAATACGATTAAAAG -3'), and *nog1* (5'- GAAAGATTTCGAGGTGATTTTCC -3' and 5'- AGATAAAAATCTCAGAACCTTGAATC -3') genes, in addition to *Smed-admp*, from asexual cDNA. Gene sequences were determined using 5′ and 3′ RACE PCR (Ambion) for all genes. PCR products from all genes and the control gene *unc-22* from *C. elegans* were cloned into the pPR244 RNAi expression vector using Gateway recombination reactions as previously described (*1*). RNAi experiments were performed by feeding the animals a mixture of liver and bacteria expressing dsRNA (*1*). Twenty milliliters of bacterial culture was pelleted and resuspended in 60 µl of liver. Animals were fed on day 0, day 4, and day 7 and amputated on day 8 for *bmp4*, *smad1*, *smad4* and *nog1* RNAi regeneration experiments. For *bmp4*, *smad1*, *smad4*, and *nog1* RNAi homeostasis experiments, animals were fed on day 0, day 4, day 7, day 14, and fixed on day 21. In all *admp* RNAi experiments, animals were fed on day 0, day 4, day 7, and fed at least five more times, once weekly. For *admp* RNAi regeneration experiments, animals were amputated one day after the final feeding. For *admp* RNAi homeostasis experiments, animals were fed weekly for 3+ months and fixed one week after the final feeding.

Phylogenetic Analyses

BLAST searches were performed on assemblies of the *L. gigantis* (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html)*, H. robusta* (http://genome.jgi-psf.org/Helro1/Helro1.home.html), and *C. teleta* (http://genome.jgi-psf.org/Capca1/Capca1.home.html) genomes to identify putative *admp* gene sequences in these species. These sequences, along with *Smed-admp* and several deuterostome TGFβ genes, were then aligned using CLUSTALW (*2, 3*). The alignments were trimmed using GBlocks (*4*) allowing for smaller final blocks, gap positions within the final blocks, and less strict flanking positions. Neighbor joining analyses were performed using Phylip (*5*) with default parameters and 500 bootstrap replicates. Maximum likelihoods were calculated using PhyML (*6*) with the

WAG model of amino acid evolution, 4 substitution rate categories, proportion of invariable sites and γ distribution parameter estimated from the dataset, and 100 bootstrap replicates. Bayesian analyses were performed using MrBayes (*7, 8*). Two chains were started and allowed to run for 10 million generations, 1 tree was sampled every 100 generations, and the first 7,500 trees were discarded as burn-in.

In Situ Hybridizations

Whole-mount *in situ* hybridizations and fluorescence in situ hybridizations (FISH) were performed as described (*9*).

qPCR

Total RNA was isolated from control and *admp(RNAi)* animals. cDNA was prepared using oligo-dT primer and quantitative PCR (qPCR) was performed using SYBR Green (Applied Biosystems). Data were normalized to the expression of GAPDH as previously described (*10*). *bmp4* specific primers were used to evaluate gene expression (5′-AAATGTACGGATTTTGGAGGAATA -3′ and 5′- GTAGGCAAAGGAGCTTTATTACCA -3′). Samples without reverse transcriptase were used as the negative control template.

Immunostaining

Immunostainings were performed as previously described (*11*) using tyramide signal enhancement.

Supplemental References

S1. P. W. Reddien, A. L. Bermange, K. J. Murfitt, J. R. Jennings, A. Sánchez Alvarado, *Dev Cell* **8**, 635 (May, 2005).
S2. D. G. Higgins, *Methods Mol Biol* **25**, 307 (1994).
S3. J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res* **22**, 4673 (Nov 11, 1994).
S4. J. Castresana, *Mol Biol Evol* **17**, 540 (Apr, 2000).
S5. J. Felsenstein, *Cladistics* **5**, 164 (1989).
S6. S. Guindon, O. Gascuel, *Syst Biol* **52**, 696 (Oct, 2003).
S7. J. P. Huelsenbeck, F. Ronquist, *Bioinformatics* **17**, 754 (Aug, 2001).
S8. F. Ronquist, J. P. Huelsenbeck, *Bioinformatics* **19**, 1572 (Aug 12, 2003).
S9. B. J. Pearson *et al.*, *Dev Dyn* **238**, 443 (Feb, 2009).
S10. G. T. Eisenhoffer, H. Kang, A. Sánchez Alvarado, *Cell Stem Cell* **3**, 327 (Sep 11, 2008).
S11. P. W. Reddien, N. J. Oviedo, J. R. Jennings, J. C. Jenkin, A. Sánchez Alvarado, *Science* **310**, 1327 (Nov 25, 2005).